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Expression of the oncogenes *mil* and *ras* abolishes the *in vivo* differentiation of mammary epithelial cells

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Three carcinoma-associated oncogenes, two of which have been strongly implicated in human mammary tumorigenesis, have been introduced into a novel mouse mammary epithelial cell line, EF43, that retains many differentiated functions. The effect of oncogene expression upon classical transformation parameters as well as parameters specific for mammary epithelial cells such as growth in three-dimensional collagen matrices and the ability to repopulate the cleared mammary fat pad and to form alveolar structures *in vivo* has been investigated. Expression of v-*myc* in EF43 cells results in no obvious phenotypic changes, and does not confer tumorigenic potential upon the cells. Expression of v-Ha-*ras* confers upon EF43 cells the ability to grow rapidly, grow in an anchorage-independent manner, results in tumor formation in nude and syngeneic animals, abolishes their ability to repopulate the mammary gland and, instead, results in rapid induction of anaplastic tumors. The v-*mil* oncogene, an avian homolog of the mouse v-*mht* and human c-*raf* oncogenes, previously thought to be non-transforming in the absence of a co-operating oncogene, transforms EF43 cells, allowing them to grow in an anchorage-independent manner, form tumors in nude mice and abolishes their ability to repopulate the cleared mammary fat pad. In contrast to v-*ras*, however, the tumors arising from v-*mil* expression have a differentiated morphology, typical of adenocarcinomas. Thus, different oncogenes show varying degrees of inhibition of the differentiation of mammary epithelial cells *in vivo*.

Introduction

Neoplastic transformation of the mammary gland proceeds via a multistep pathway involving the tumorigenic conversion of one or a number of mammary cell types. The identity of the cell types involved in this process, their interaction during the conversion and the identity of the cellular genes which are deregulated is open both to speculation and investigation.

Transfection of DNA from the human mammary tumor derived cell line MCF-7 into NIH/3T3 fibroblasts has identified potential mammary oncogenes (1). Additionally, the Ha-*ras* oncogene has been found activated or overexpressed in some human mammary tumors (2–4) and a human mammary tumor cell line (5,6). Two *erb-B* related genes, the human epidermal growth factor (EGF*) receptor gene (7,8) and the *neu* oncogene (9) have been found amplified in human mammary carcinoma derived cell lines. More recently, the *neu* oncogene has been shown to be amplified in primary mammary tumors (10). Similarly, the c-*myc* gene has been found to be amplified (11) and overexpressed in some human primary breast carcinomas (12).

In the mouse, depending upon strain, either plaques or hyperplastic alveolar nodules (HANs) represent the first visible preneoplastic lesion. HANs, and less frequently plaques, may eventually progress to overt mammary tumors (13). During this progression a number of genes may become activated including the putative mammary oncogenes, *int-1* (14), *int-2* (15), *int-3* (16) and *int-H* (17). Two of these oncogenes have been shown to be activated early in mammary tumorigenesis (18,19).

Although there is a correlation between expression of oncogenes and the transformed phenotype, a causal relationship between expression and transformation of the normal mammary gland has not yet been demonstrated. In order to determine whether any of the known oncogenes implicated in mammary tumorigenesis are causally involved, we have introduced the activated cellular Ha-*ras* oncogene, the viral *ras* and *myc* oncogenes, and the v-*mil* oncogene—the murine homolog of which has been implicated in carcinoma formation (20,21)—into a normal mouse mammary epithelial cell line, EF43. Although this mammary cell line is able to divide indefinitely in culture, it retains many of the properties of normal mammary epithelial cells, including the ability to repopulate the cleared mammary fat pad and respond to the mammotropic stimuli of pituitary hormones.

The effects of the expression of these genes on classical parameters of transformation such as morphology, anchorage-independent growth and tumorigenicity in nude and syngeneic mice have been investigated. In addition, we have examined the consequences of the expression of these oncogenes upon some properties of differentiated mammary epithelial cells such as the ability to grow in collagen matrices, induction of casein in response to treatment with lactogenic hormones, and repopulation of cleared mammary fat pads. Finally we determined the effects of these oncogenes on the dependence of mammary epithelial cells upon EGF, insulin and serum for growth.

Our data suggest that the introduction and expression of the activated c-Ha-*ras* or v-Ha-*ras* and the v-*mil* oncogene in normal mouse mammary epithelial cells results in tumorigenic conversion and interference with the *in vivo* differentiation of the cells. In contrast, expression of the v-*myc* gene in these cells does not appear to interfere with normal mammary differentiation in terms of the parameters we have measured.

Materials and methods

Plasmid vectors

A 1.7-kb *Bam*HI–*Bgl*II fragment containing the v-Ha-*ras* gene from Harvey murine sarcoma virus (pHaSV; 22) and a 1.5-kb *Bam*HI–*Ava*I fragment containing

*Abbreviations: EGF, epidermal growth factor; HANs, hyperplastic alveolar nodules.

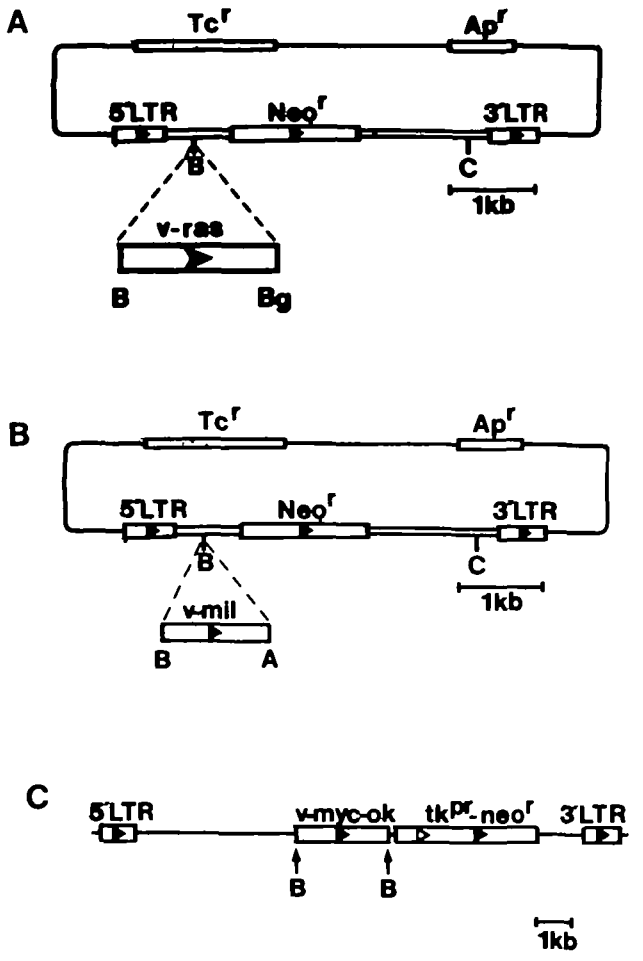

Fig. 1. Schematic diagrams of the plasmids pMMCV-neo (26), pZIPras and pZIPmil. pZIPras and pZIPmil were constructed as outlined in Materials and methods. *Bam*HI sites are designated B, *Bgl*II sites are designated Bg, *Ava*I sites are designated A, *Cla*I sites are designated C. The herpes simplex thymidine kinase promoter is marked tk^{pr} and the direction of the promoter is indicated by open arrows. The neomycin-resistance gene from Tn5 is marked neo^r. The tetracycline- and ampicillin-resistance genes are indicated Tc^r and Ap^r respectively. Direction of gene transcription is indicated by closed arrows.

the v-*mil* gene of the MH-2 virus (pMH-2-BH; 23) were inserted in the *syn* orientation into the *Bam*HI site of pZIPneoSV(X) (24) to generate the plasmids pZIP-*ras* and pZIP-*mil* respectively (Figure 1A and B). The v-*myc* containing pMMCV-neo plasmid (Figure 1C; 25) was kindly provided by B.Vennström, Heidelberg, FRG. The pSV2neo-EJ plasmid was derived from pSV2-neo (26) by the insertion of a 6.6-kb *Bam*HI fragment of cloned genomic DNA from the plasmid pEJ (27) into the unique *Bam*HI site of pSV2-neo (N.E.Hynes, Ludwig Institute, Bern, unpublished).

Cell culture, transfections and retroviral infections

EF43 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin; 1 μg/ml insulin and 5 ng/ml EGF. Cells were transfected with the plasmid pSV2neo-EJ essentially as outlined previously (28). Stable, neomycin-resistant clones were selected in 200 μg/ml G418. Retroviral infection after transient transfection of Ψ2 cells (29) was carried out as described previously (30).

Test for growth factor dependence

Suspensions of single cells at a density of 1×10^4 were seeded in a 6-cm plate in normal medium. The medium was changed 24 h later to medium containing combinations of 0.5% fetal serum, EGF and insulin. Duplicate cultures were counted in a Coulter Counter at this time and 6 days later.

Growth in collagen

Cells were established in collagen matrix cultures essentially as described by Jones and Hosick (31). Briefly, 1×10^5 cells were resuspended in 10 μl of medium and mixed with 80 μl of rat-tail collagen (1 mg/ml, 6 μl/ml 6.27 mM acetic acid) and 10 μl setting solution (6 × RPMI, 0.075 N NaOH, 0.0245 g/ml $NaHCO_3$). This mixture was plated into a collagen-coated well of a 12-well cluster plate and left to set 15 min at 37°C. Two milliliters of medium were then added to each well, the collagen matrices detached from the Petri dish and the collagen cultures incubated for 2–4 weeks under normal conditions with regular medium changes.

Assay for growth in soft agar

Cells (1×10^5) from a single cell suspension were plated into 8 ml of DMEM containing antibiotics, 10% fetal calf serum and 0.35% bacto-agar. This was layered into 6-cm Petri dishes precoated with a bottom layer of 2 × soft agar. The soft agar dishes were incubated in moist chambers under normal culture conditions. The appearance of colonies of cells growing in the soft agar was noted after 2 weeks.

Assay for tumorigenicity in nude mice

Cells (1×10^6) from a single cell suspension were washed twice in serum- and pyrogen-free DMEM before being resuspended in 200 μl of the same medium. This was injected s.c. into 2-week-old Nu/Nu mice under aseptic conditions. The mice were observed once a week for the appearance of tumors at the site of injection.

Repopulation of the cleared mammary fat pad

Cells ($1-3 \times 10^5$) in serum-free Hanks' medium were injected in a 10-μl volume into the cleared no.4 fat pads of 18-day-old female BALB/c mice using a 30-gauge Hamilton Syringe (32).

Pituitary isograft procedure

Pituitary isografts were performed using the method of ref. 33. Pituitary donors were killed by cervical dislocation, the pituitary gland removed and inserted into an 18-gauge trocar. Two pituitaries were inserted into the spleen of anesthetized recipient mice. The hormonal activity of the isografts was assessed by evaluating vaginal smears and by microscopic examination of the isograft and mammary glands.

Preparation of whole amounts of mammary fat pads

After ventral incision, the no. 4 mammary glands were removed with the skin flap attached and fixed in acetic acid/ethanol (1:3 v/v) for at least 1 h. The glands were washed in 70% ethanol and then in distilled water before being dissected away from skin. The glands were stained in Alum Carmine solution for at least 4 h, washed with ethanol and then destained in methyl salicylate.

Expression of oncogene products

To test for expression of $p21^{ras}$ in the EF43J series and in EF43zip-ras-pop, cell membrane preparations were made as outlined previously (30). The proteins were separated on a 17% SDS–polyacrylamide gel, electroblotted to nitrocellulose and reacted with the appropriate antisera and visualized as previously described (30). Anti-$p21^{ras}$ was kindly donated by Dr R.Sweet (Smith, Kline and French Laboratories, Philadelphia, PA). Radiolabeling of cellular protein with $[^{35}S]$-methionine, extraction and immunoprecipitation of the v-*myc* and v-*mil* proteins was performed as outlined previously (28) using anti-*myc* serum (Cambridge Research Biochemicals) and anti-*mil* serum, kindly supplied by J.Ghysdael (Institut Pasteur, Lille, France).

Results

Characterization of EF43 cells

The mammary epithelial cell line EF43 was derived as a clonal outgrowth from the mammary gland of an irradiated BALB/c female mouse as previously described (34). EF43 cells retain most of the differentiation characteristics of normal mammary epithelium including the formation of normal ductal structures in the cleared mammary fat pads of syngeneic mice (Figure 4A). Further, when these mice receive pituitary isografts, the ductal structures form alveolar outgrowths at a frequency of 60% (Figure 4A, inset), demonstrating that EF43 cells are capable of responding to the mammotropic effects of pituitary hormones. EF43-derived outgrowths differentiate in pregnant mice and produce milk, regressing to normal ductal structures following weaning of the litter. The EF43 cell line is heterogeneous with respect to keratin expression using the DAKO rabbit anti-human stratum corneum keratin antiserum (results not shown). Heterogeneity in keratin expression has been reported for another mouse mammary cell line that produces mammary outgrowths (35). Taken together, these data suggest that EF43 cells may be mammary progenitor cells.

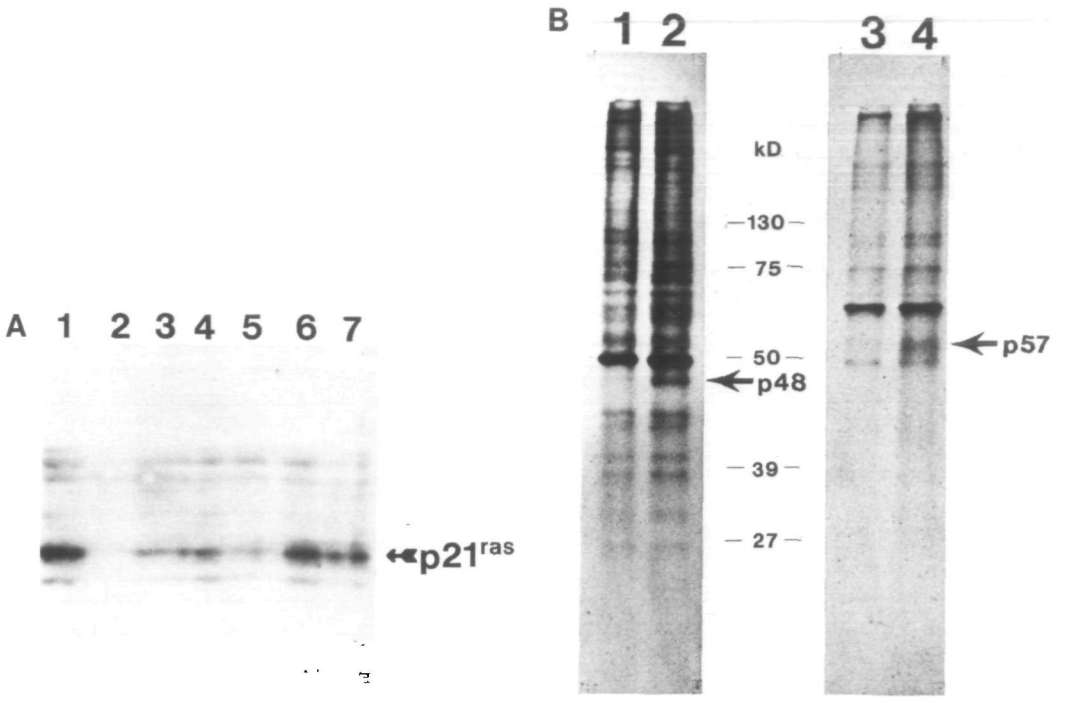

Fig. 2. Expression of the *ras*, *mil* and *myc* oncogene proteins. (**A**) Membrane proteins from EF43 cells (**lane 2**), EF43 cells infected with ZIPras virus (**lane 1**) and five clones of EF43 cells transfected with pSV2neo-EJ, J1 (**lane 3**); J2 (**lane 4**); J3 (**lane 5**); J6 (**lane 6**); and J7 (**lane 7**) were separated on a 14% SDS-polyacrylamide-gel. After transfer to nitrocellulose, *ras*-specific proteins were visualized by treatment of the filter with anti-$p21^{ras}$ followed by incubation with iodinated protein A. The $p21^{ras}$ protein is marked. (**B**) EF43 cells infected with ZIPneoSV(X) (**lanes 1** and **3**), ZIP-*mil* (**lane 2**) or ZIP-*myc* (**lane 4**) virus were cultured in DMEM with 10^{-5} M methionine plus 200 μCi [^{35}S]methionine for 3 h. Total cell proteins were extracted and immunoprecipitated with anti-*mil* (**lanes 1** and **2**) or anti-*myc* (**lanes 3** and **4**) serum. The anti-*mil* serum specifically precipitates a protein of 48 kd. The anti-*myc* serum specifically precipitates $p57^{myc}$. The positions of reference standard proteins are marked.

Introduction and expression of the oncogenes ras, myc and mil in the EF43 mammary cell line

The plasmid pSV2neo-EJ containing the activated c-Ha-*ras* gene and the gene for neomycin resistance was transfected into the EF43 cell line. G418-resistant clones were selected and grown into mass culture for Southern blot analysis. Five cell clones (J1, J2, J3, J6 and J7) contained the transfected plasmid and were chosen for further analysis (data not shown).

Retroviral infection has become an attractive method for the introduction of genes into cells (for review see 36). Among the reasons for this are the relatively high efficiency of gene transfer as well as the stability, integrity and low copy number of the acquired proviral DNA. Infection of EF43 cells with virus produced upon transfection of the Ψ2 MoMLV packaging cell line (29) with pZIPneoSV(X) proceeds with high efficiency (30; N.B. EF43 is referred to as #43). We therefore sought to use retroviral vectors as an efficient means of introducing oncogenes into the EF43 cell line.

The pZIPneoSV(X), pZIP*ras*, pZIP*mil* and v-*myc*-containing pMMCV-neo plasmids (Figure 1) were independently transfected into the Ψ2 MoMLV packaging cell line (29) and the virus produced used to infect EF43 cells. G418-resistant populations of EF43 cells independently infected with *ras*-containing virus (EF43zip-*ras*-pop), the *mil*-containing virus (EF43zip-*mil*-pop), and the empty ZIPneoSV(X) vector (EF43zip-pop) were established. In addition, a clone of EF43 cells infected with *myc*-containing virus (EF43*myc*) was obtained. Southern blot analysis of the infected EF43 cell populations showed that each has acquired the appropriate viral DNA (data not shown).

Expression of introduced oncogenes in EF43 cells

Expression of the *ras* oncogene in the pSV2neo-EJ-transfected EF43 cell lines and the ZIP-*ras*-infected EF43 cell population was analysed by Western blotting of crude membrane extracts (Figure 2A). Little if any $p21^{ras}$ is detected in the EF43 cell line

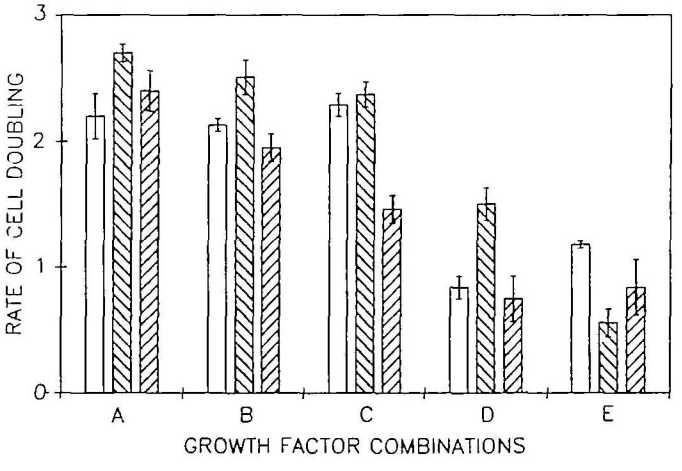

Fig. 3. Graphic representation of growth factor dependence of EF43 cells. The rate of growth from duplicate points of cells grown logarithmically over a period of 6 days was determined in medium containing (**A**) 0.5% fetal calf serum, 1 μg/ml insulin and 5 ng/ml EGF; (**B**) 0.5% fetal calf serum and 5 ng/ml EGF; (**C**) 0.5% fetal calf serum and 1 μg/ml insulin; (**D**) 0.5% fetal calf serum; (**E**) 5 ng/ml EGF and 1 μg/ml insulin. Growth rate of EF43 cells infected with the ZIPneoSV(X) virus is shown by open blocks, of EF43 cells infected with the ZIP-*ras* virus, EF43zip-*ras*-pop, by descending shading (left to right) and of EF43 cells infected by the ZIP-*mil* virus, EF43zip-*mil*-pop, by ascending shading (left to right). The error bars indicate the deviation of the actual data from the plotted mean.

(lane 2). Relatively high levels of p21^{ras} are detectable in both the virus-infected EF43zip-*ras*-pop (lane 1), and the five pSV2neo-EJ-transfected cell clones J2 (lane 4), J6 (lane 6) and J7 (lane 7) and lower levels in cell clones J1 (lane 3) and J3 (lane 5). The expression of the *mil* and *myc* proteins was investigated by immunoprecipitation. A *mil*-specific protein of 48 kd is detected in the ZIP-*mil*-infected population of EF43 cells (Figure 2B, lane 2) which is not present in control ZIPneoSV(X) virus-infected cells (Figure 2B, lane 1) or non-infected EF43 cells (data not shown). This protein is smaller than that seen in MH2 virus-infected cells (p100) presumably since it lacks the fused *gag*-specific sequences (37). A similar *raf*-related protein of 48 kd has been previously described (38). Expression of the *myc* gene in the MMCV-neo virus-infected cell line EF43-*myc* was also investigated. Using anti-*myc* serum, p57^{myc} is detectable in EF43-*myc* cells (Figure 2B, lane 4) but not in ZIPneoSV(X) virus-infected EF43 cells (Figure 2B, lane 3). The endogenous c-*myc* protein is expressed at very low levels and is undetectable in this experiment. Expression of p57^{myc} in the EF43-*myc* cells was also demonstrated by Western blotting (data not shown). Thus, expression of the introduced oncogenes can be detected in each of the *ras*-, *mil*- and *myc*-infected cell lines.

Effect of oncogene expression upon growth of the EF43 cell line

Oncogenes are known to alleviate dependence upon growth factors (for reviews see 39,40). We therefore sought to investigate the effects of expression of oncogenes upon growth factor dependence of the EF43 cell line. To eliminate any effects of clonal variation we compared only the ZIP-*ras* and ZIP-*mil* virus-infected populations with the ZIPneo SV(X) virus-infected EF43 cell line. The rate of growth of logarithmically growing control EF43zipSV(X) cells and EF43zip-*ras*- and EF43zip-*mil*-infected populations was determined over a 6-day period. EF43 cells grow in the presence of 0.5% fetal bovine serum supplemented with either EGF or insulin or both (Figure 3). In the absence of both EGF and insulin, the growth rate of the EF43 cells decreases >50%, indicating a requirement for these factors. Indeed, EF43 cells grow better in medium containing both EGF and insulin but lacking serum than when grown in serum but lacking both growth factors. In the presence of serum, expression of the Ha-*ras* oncogene increases the growth rate of EF43 cells and reduces their dependency upon EGF and insulin. However, the expression of p21*ras* appears to have rendered the EF43 cells more dependent on some factor(s) present in serum. This factor(s) seems to co-operate with *ras* expression to increase the growth rate of the EF43 cells. In contrast, introduction of the v-*mil* oncogene results in a slight reduction in growth rate in almost all of the growth conditions.

Effect of oncogene expression upon morphology of the EF43 cell line

We investigated the morphology of the EF43 cell line, the populations of virus-infected cells (EF43zip-*mil*-pop, EF43zip-*ras*-pop, EF43zip-pop) and the clones generated either by transfection (pSV2neo-EJ-transfected J series) or virus infection (EF43*myc*) on plastic. At low cell density, no gross difference in morphology is observed between the EF43 cell line and the EF43 cell line containing *ras*, *mil* or *myc* when cultured on plastic. At confluence, however, EF43 cells expressing the *ras* oncogene reach a higher cell density and are metabolically more active than the parental EF43 cells or EF43 cells expressing the *mil* or *myc* oncogenes (data not shown).

Non-transformed mammary gland cells grown in collagen will form three-dimensional tubular structures reminiscent of mammary gland ducts (31,41). EF43 cells also display this property (R.Ullrich, unpublished observations). Expression of the *ras*, *mil* or *myc* oncogenes does not appreciably affect the morphology of EF43 cells grown in collagen gels (data not shown).

Effect of oncogene expression upon the anchorage-independent growth and tumorigenicity in nude mice of the EF43 cells

EF43 cells do not grow in an anchorage-independent manner (Table I) and are not tumorigenic in nude mice. Introduction and expression of the *ras* oncogene either by transfection (J1, J2, J3, J6 and J7) or by infection with a *ras*-containing virus (EF43zip-*ras*-pop) confers upon them the ability to grow in an anchorage-independent manner (Table I). Further, the EF43zip-*ras*-pop cells rapidly induce tumors in nude mice and are also tumorigenic in syngeneic animals (Table I). The transfected *ras* gene is present in DNA extracted from a nude mouse tumor arising from the J3 cell line (data not shown) indicating the involvement of the *ras* oncogene in tumorigenesis. Introduction of the v-*mil* oncogene into EF43 cells also confers upon them the ability to grow in an anchorage-independent manner. However, introduction and expression of v-*myc* does not result in anchorage-independent growth of EF43 cells (Table I).

Table I. Effects of oncogenes introduced into EF43 cells upon anchorage-independent growth, *in vivo* repopulation, and tumorigenicity

Cell line		Growth in soft agar[a]	Tumorigenicity in nude mouse[b]	Repopulation of cleared fat pad[c]		
				No. of mammary outgrowths	No. of tumors	Latency (days)
EF43	Non-transfected	−ve	0/2 after 7 weeks			
J1	*ras* transfected	30%	1/2 after 6 weeks	0/16	15/16[e]	40
J2	*ras* transfected	13%	2/2 after 2 weeks	0/16	16/16[e]	23
J3	*ras* transfected	40%	2/2 after 3 weeks	0/16	16/16[e]	17
J6	*ras* transfected	12%	2/2 after 2 weeks	0/16	16/16[e]	15
J7	*ras* transfected	25%	2/2 after 3 weeks	NT		
EF43zip-pop	Infected	−ve	NT	7/16[d]	0/16[g]	
EF43zip-*ras*-pop	Infected	22%	2/2 after 5 weeks	0/16	16/16[e]	38
EF43zip-*mil*-pop	Infected	33%	NT	0/16	9/16[f]	54
EF43*myc*	Infected	−ve	NT	4/16[d]	0/16[g]	

[a]1×10^5 cells plated.
[b]1×10^6 cells injected.
[c]2×10^5 cells injected.
[d]Normal ductal outgrowth.
[e]Undifferentiated morphology.
[f]Differentiated morphology.
[g]Animals observed for 54 days.
NT, not tested.

Effect of oncogene expression upon the repopulation of the cleared normal mammary fat pad

Normal ductal outgrowths can be observed when EF43 cells are reimplanted into the cleared mammary fat pad (Figure 4A). Reintroduction of EF43 cells infected with the empty ZIPneoSV(X) virus, EF43zip-pop, also resulted in normal ductal repopulation in 7 out of 16 (44%) mice (Table I). However, introduction and expression of the Ha-*ras* oncogene both in the J series and in the EF43zip-ras-pop totally abolishes this repopulation and instead results in the rapid and efficient (100%) induction of tumors (Table I). These tumors are histologically undifferentiated (Figure 4B,a). The introduction of the v-*mil* oncogene into EF43 cells also leads to rapid tumor formation (Table I). However the tumors derived from expression of the *mil* gene are histologically highly differentiated (Figure 4B,b). Expression of the v-*myc* gene does not interfere with the normal repopulation of the mammary gland and these cells are not tumorigenic (Table I). When mice carrying normal ductal outgrowths derived from the EF43 cells, the EF43zip-pop cells or the EF43*myc* cells received pituitary isografts, the ductal outgrowths became alveolar, indicating a normal differentiation response to pituitary hormones (Figure 4A, inset).

Discussion

Relatively little is known about the effects of oncogenes on epithelial cells. Among the reasons for this are the difficulties in obtaining and culturing primary epithelial cells, and the difficulties involved in the introduction of oncogenes into these cells which are generally refractile to gene transfer using conventional techniques. The EF43 cell line divides indefinitely in culture, differentiates *in vivo* and is readily transfectable and infectable (30). Since the EF43 cell line is also non-tumorigenic, it appears to represent the best available cell line for studying the effects of cloned oncogenes upon differentiation parameters of normal mammary epithelium, and the potential involvement of such oncogenes in mammary tumorigenesis. We have introduced the Ha-*ras*, v-*mil* and v-*myc* oncogenes independently into this cell line and measured the effects of expression of these genes upon a range of parameters.

Our results were not affected by the method of introduction of the genes (transfection versus infection) or whether cell clones or populations were analysed. The introduction and expression of either the activated cellular or viral homolog of the Ha-*ras* oncogene resulted in a tumorigenic conversion grossly characterized by the ability to grow in an anchorage-independent

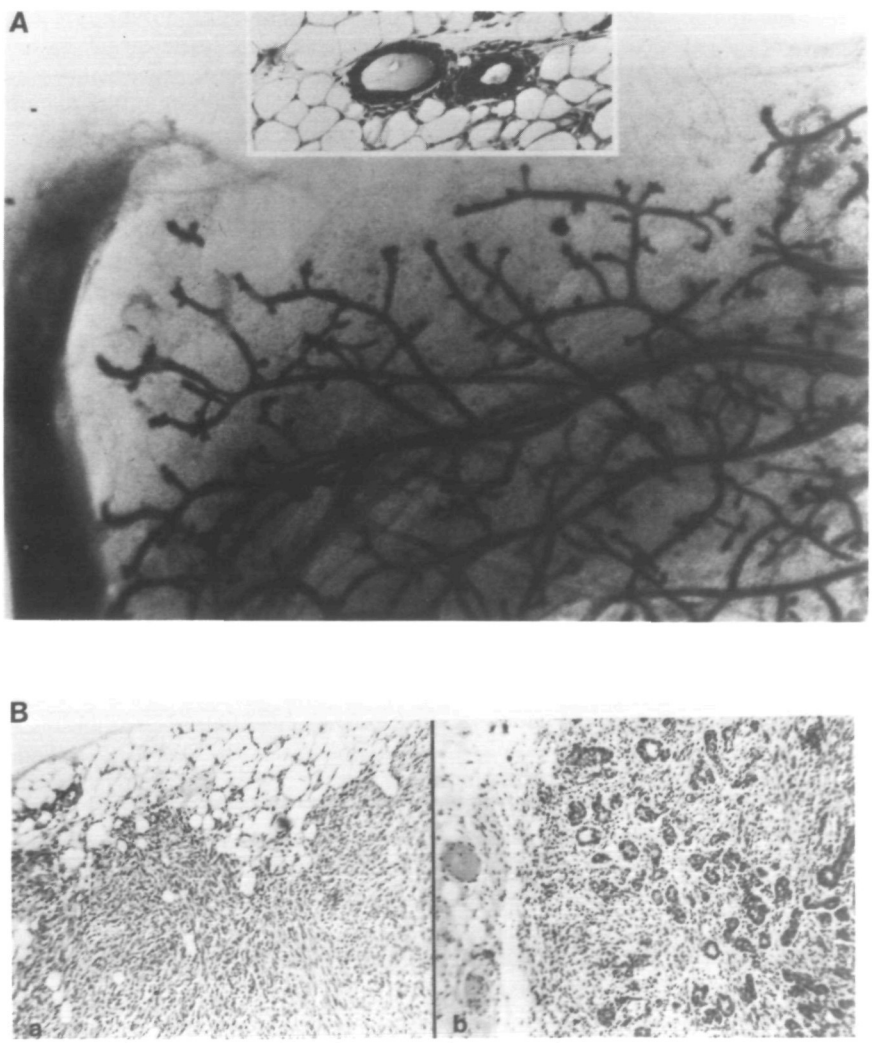

Fig. 4. (A) Mammary gland repopulated with EF43 cells at passage 18. The inset shows the histology of alveolar differentiation after pituitary isograft. (B) Histology of tumors from reimplantation into the cleared mammary fat pads of BALB/c mice. (a) A poorly differentiated anaplastic tumor formed after repopulation with the *ras* transfected clone J1. The other *ras*-transfected cell clones and *ras*-infected cell population also formed anaplastic tumors of similar histology (data not shown). (b) A well-differentiated adenocarcinoma formed by EF43 cells (EF43zip-*mil*-population) infected with the v-*mil*-containing retrovirus.

manner and the formation of tumors in both nude and syngeneic animals (Table I). Similar results have been obtained after transfection of another mammary cell line, NMuMG, with the Ha-*ras* oncogene (42). Since our interest is to determine the effects of oncogenes potentially involved in mammary tumorigenesis upon mammary gland cell growth and differentiation, we chose to investigate parameters relevant to the mammary gland.

Although expression of *ras* does not affect the morphology of the EF43 cells either on plastic or within collagen matrices, the growth rate of the EF43 cells is increased and their requirement for EGF and insulin is reduced (Figure 3), implying that *in vivo* such transformed cells are no longer responsive to growth factor control of proliferation. However, expression of $p21^{ras}$ does not reduce the requirement for serum, indicating that serum may contain additional factors which co-operate with $p21^{ras}$ to increase the overall growth rate of the EF43 cells. This factor(s) is clearly not EGF or insulin. Recently it has been shown that a factor(s) present in serum may enhance the emergence of tumor cells upon transfection of the Ha-*ras* oncogene into fibroblasts. This factor does not appear to be a known growth factor (43). Expression of Ha-*ras* prevents the *in vivo* parameter of repopulation of the cleared mammary fat pad by the EF43 cells, and instead results in the rapid formation of anaplastic tumors. Anaplastic tumors are indicative of the later stages of mammary tumor progression. The EF43 cells infected with retrovirus lacking an oncogene are still able to participate in normal repopulation of the cleared mammary fat pad, indicating that expression of the Ha-*ras* oncogene disrupts normal differentiation.

The v-*mil* gene was originally isolated from the avian carcinoma retrovirus MH2 which also carries a second oncogene, v-*myc* (23). Deletion mutants of MH2 suggest that v-*myc* is the primary oncogene of this virus whilst v-*mil* has auxilliary oncogenic functions (44). In contrast, the murine homolog, v-*raf*, found in the virus 3611-MSV, efficiently transforms established (20) as well as primary epithelial cells and established fibroblasts (21). In view of the involvement of the *mil/raf* oncogene family in carcinomas (20,21), we have investigated the effect of introduction and expression of the v-*mil* oncogene in EF43 cells. A *mil*-related protein of 48 kd is detected in EF43 cells infected with the v-*mil*-containing virus. This protein lacks the *gag* portion of $p100^{gag\text{-}mil}$ found in MH2 virus-infected cells and has the same mobility as a *raf*-related protein (38). Expression of this protein results in transformation of mammary epithelial cells. The v-*mil* oncogene, like Ha-*ras*, induced anchorage-independent growth in the EF43 cells (Table I) without any gross changes in cell morphology. In contrast to *ras* expressing cells, this is not accompanied by an increase in growth rate or an alleviation of growth factor dependence since EGF and insulin are required for growth (Figure 3). Expression of v-*mil* abolishes the ability of the EF43 cells to repopulate the cleared mammary fat pad and instead leads to the formation of differentiated adenocarcinomas (Figure 4). Thus this truncated form of the *mil* gene has a transforming activity in mammary epthelial cells and could play a role in early stages of transformation of the mammary gland.

The *myc* gene has been implicated in both human (11,12) and mouse (45) mammary carcinogenesis. Introduction and expression of the v-*myc* by infection with the MMCV-neo retrovirus (25) did not cause transformation of the EF43 cells, as judged by the parameters we have tested. In accordance with the lack of transformation, the ability of these cells to repopulate the cleared mammary fat pad and respond to pituitary hormones was unaffected (Table I). Recently it has been shown that *myc* has no apparent effect on established fibroblasts of mammalian (46,47) or avian (48) origin. It is possible that the levels of v-*myc* expression may not be sufficient to affect the EF43 cells. However, using a pan-*myc*-specific antiserum we were only able to detect v-*myc*, indicating a much higher expression than of c-*myc*. Since EF43 cells grow indefinitely in culture, it is probable that some immortalizing event has already occurred. This may have rendered this cell line refractile to the effects of expression of the *myc* oncogene, which is known to immortalize primary cells (49). The v-*mil* oncogene has previously been regarded as a potentiating oncogene, having no intrinsic transforming ability. However, EF43 cells expressing v-*mil* are clearly tumorigenic *in vivo*. If a *myc*-like event had already occurred in these cells, v-*mil* may potentiate that effect resulting in neoplastic characteristics.

Taken together, these data suggest that mammary tumorigenesis proceeds by a number of stages, each of which is accompanied by a concomitant loss of differentiation. Since the cell line that we have chosen shows indefinite growth *in vitro*, it is not 'normal'; however, the EF43 cell line *is* still responsive to factors that control mammary gland growth and differentiation. Introduction and expression of a *myc* oncogene does not affect this response. Thus although we cannot be certain whether indefinite growth (immortalization) is a prerequisite for mammary tumorigenesis, it is not sufficient *per se* to cause the development of mammary neoplasia since it does not appreciably disrupt the differentiative response of the mammary gland. The activation of a cellular oncogene, functionally similar to *mil/raf*, in cells that have already acquired the ability to proliferate indefinitely, leads to a relatively differentiated tumor, the growth of which is still dependent upon growth factors. Presumably the next stage(s) in the progression towards overt mammary tumorigenesis is the loss of growth control, a stage that can be partially achieved by activating the *ras* oncogene. This would completely free the mammary cell from growth control and contribute to its invasiveness.

Our description of the molecular events that occur in mammary tumorigenesis would accommodate earlier observations that, at least in some mouse strains, the mammary tumor is reversible—appearing during pregnancy when hormonal growth stimulation is present, then regressing (growth factor responsive tumor) and after three or four pregnancies becoming pregnancy (and growth factor) independent (18). Rapid growth favors the accumulation of mutations required for the progression to the overt mammary tumor. In this light it is interesting that both *int-1* and *int-2* are putative embryonic growth factors (50,51) that are inappropriately expressed in mammary tumors, and may contribute to, but are probably not the primary cause of mammary tumors.

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